Studies on bacterial motility as a virulence factor and stimulation response

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論文内容要旨

Studies on bacterial motility as a virulence factor and stimulation response

(病原因子及び刺激応答としての細菌運動に関する研究)

東北大学農学研究科

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CHAPTER 1. General introduction

Bacteria occupy every corner on the planet, even where it might be extreme to mankind. Robustness and diversity of the bacterial ecosystem is achieved by their excelled adaptation, modification and resilience capable of responding variation of circumstances. These include the interaction of bacteria with the outer environment and other species around, in some cases, leading to symbiosis of multiple species or the establishment of a successful infection on the prefer animal hosts. Although bacterial infection involves various virulence factors (e.g., toxins, enzymes, adhesivity, etc.) and physiological controls including temporal survival at the viable but not culturable (VBNC) state (21), many species rely on motility.

Bacterial motility is mechanically different in different species though, many motile species exhibits flagellum-dependent motility. A thin, helical filament is rotated by a basal motor (flagellar motor) that propels the cell in liquids. The flagellar motor is fueled by an electrochemical gradient called ion motive force, which consists of membrane voltage ($\Delta\psi$) and ion concentration gradient (e.g. pH difference between the cell exterior and cell interior, ΔpH) (1). The correlation between motility and pathogenicity has been reported in many bacterial species such as *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Helicobacter pylori*, *Salmonella* and *E. coli*. Spirochetes such as *Leptospira interrogans*, the causative agent of worldwide zoonosis leptospirosis, and *Brachyspira hyodysenteriae*, the pathogen of swine dysentery, possess their flagella beneath the outer membrane. The motility of spirochetes is considered as a crucial virulence factor as with other motile bacterial pathogens (2-8).

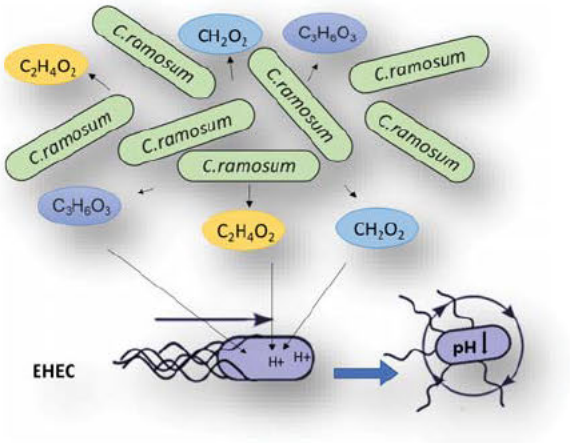
Although the majority of motile bacteria swim using flagella, the way for movement is diverse. For instance, *Mycoplasma mobile* glide over surfaces via

abundant leg-like complexes that reside on the cell surface, and *Leptospira* spp. show crawling using adhesive outer membrane molecules such as lipopolysaccharides (see Chapter 3). In either type of motility, bacteria can move towards or away from a variety of stimuli, including a concentration gradient of chemicals, light, temperature, gravity, and a magnetic field. Survival and thrive of bacteria are attributed to a reliable response to environmental stimuli is responsible.

Thus, bacterial motility depends on physiological conditions and serves as a virulence factor. Moreover, bacteria control motility in response to changes in environmental conditions. For better understanding of their pathogenesis and ecosystem, it is important to know detailed dynamic processes of individual cells by linking with their physiologies behind. To consider significance of the bacterial motility together with physiological insight in terms of various aspects, this thesis describes the following three topics.

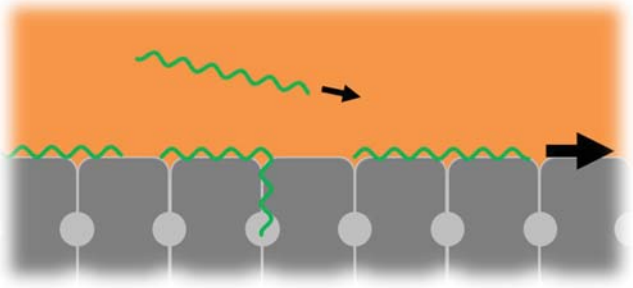
1. Bacterium-Bacterium interaction: Pathogen vs Commensal.

The first topic concerns “the regulation of bacterial motility by the biological process of other microbial species”. This topic will be given by discussing a study on the organic acids produced from the fermentation of *Clostridium ramosum*, in which showcase the regulatory effect on the motility of *E. coli* cells (9).



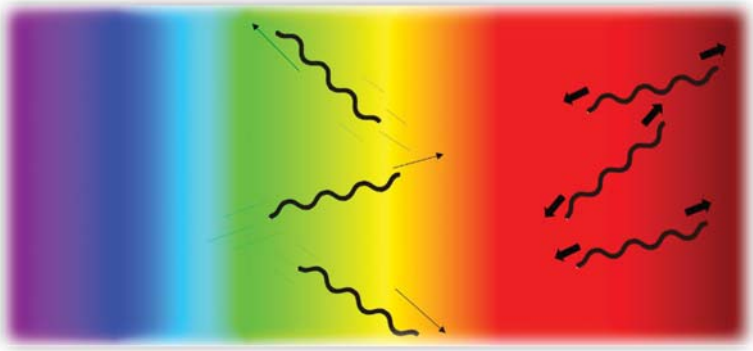
2. Bacterium-Host interaction: Pathogen vs Host

The second topic concerns “the bacterial motility to the host”. This is the continuation of the candidate’s previous studies on the inhibitory effect of mannose-binding lectin to the bacterial motility (10, 11), motility of *Salmonella* inside the egg (12), whereas the recent studies focus on the gliding motility of pathogenic *Leptospira* cells on the host animal cells in order to reveal complicated host-preference on *Leptospira* infection.



3. Bacterium response to environmental stimuli

The third topic concerns “the bacterial motility with the environmental factors”. Motility of *Leptospira* under environmental factors such as light will be discussed. In detail, a recently isolated *Leptospira* species that showcases the photoresponsivity will be introduced in the topic.



CHAPTER 2. Bacterium vs Bacterium

Effects of fermentation products of the commensal bacterium *Clostridium ramosum* on motility, intracellular pH, and flagellar synthesis of enterohemorrhagic *Escherichia coli*

As described in chapter 1, the flagellum and motility are crucial virulence factors for many pathogenic bacteria. In general, pathogens invade and translocate through motility and adhere to specific tissue via flagella. Therefore, the motility and flagella of pathogens are effectual targets for attenuation. Here, we focused on fermentation products from *Clostridium ramosum*, a harmless intestinal bacterium that commonly inhabits the human gut. High-performance liquid chromatography has shown that *C. ramosum* produces various organic acids, and the top three are formic acid (20.1 mM), acetic acid (10.2 mM), and lactic acid (12.7 mM) (Y. Koyanagi, J. Xu, and E. Isogai, submitted for publication). we show that the fermentation products of *Clostridium ramosum* decrease the intracellular pH of enterohemorrhagic *Escherichia coli* (EHEC) (Fig.1) and influence its swimming motility (Fig.2). Quantifications of flagellar rotation in individual EHEC cells showed an increase in reversal frequency and a decrease in rotation rate in the presence of *C. ramosum* fermentation products (Fig.3). Furthermore, the *C. ramosum* fermentation products affected the synthesis of flagellar filaments (Table 1). The results were reproduced by a combination of organic acids under acidic conditions. Short-chain fatty acids produced by microbes in the gut flora are beneficial for the host, e.g. they prevent infection. Thus, *C. ramosum* could affect the physiologies of other enteric microbes and host tissues. This work has been published Xu et al., 2019 (9).

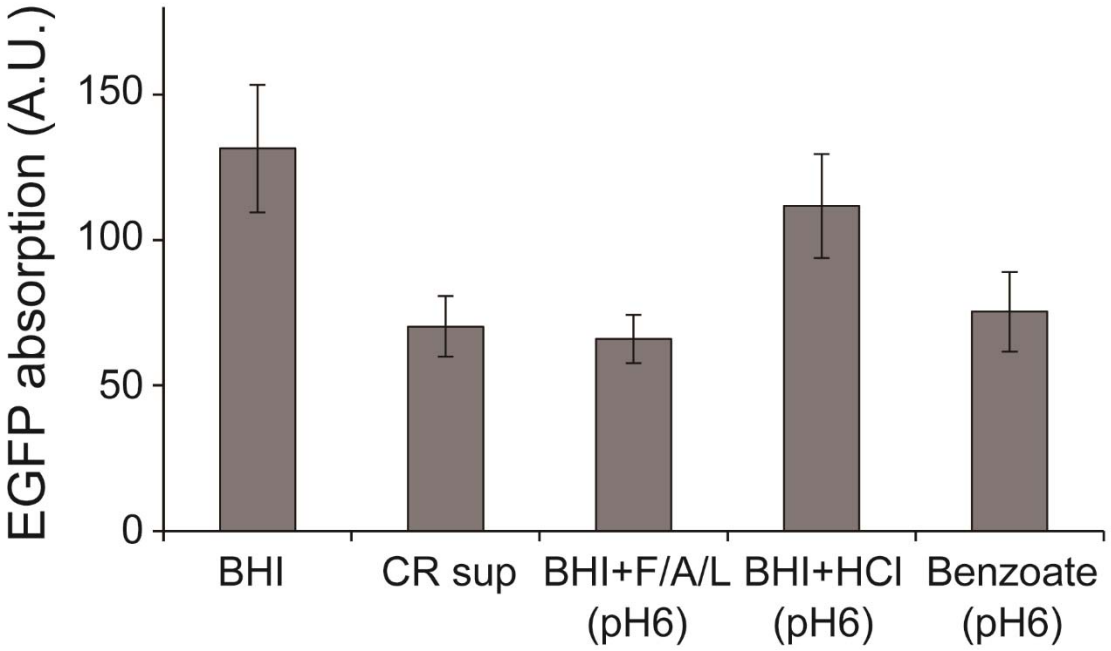


Fig. 1. Effects of fermentation products and organic acids on the intracellular pH of EHEC. Absorption at 490 nm was measured; absorption is reduced with decreasing pH. Organic acids such as benzoate permeate the cytoplasmic membrane in the protonated form and dissociate protons within the cytoplasm; decreased external pH (i.e. medium pH) facilitates the permeation of protonated acids, resulting in the reduction of intracellular pH. We evaluated the effect of *C. ramosum* supernatant on the intracellular pH of EHEC by using EGFP. Absorption of EGFP around 490 nm is decreased by decreasing the pH. (Abbreviations: CR sup: *Clostridium ramosum* supernatant; F: formic acid; A: acetic acid; L: lactic acid)

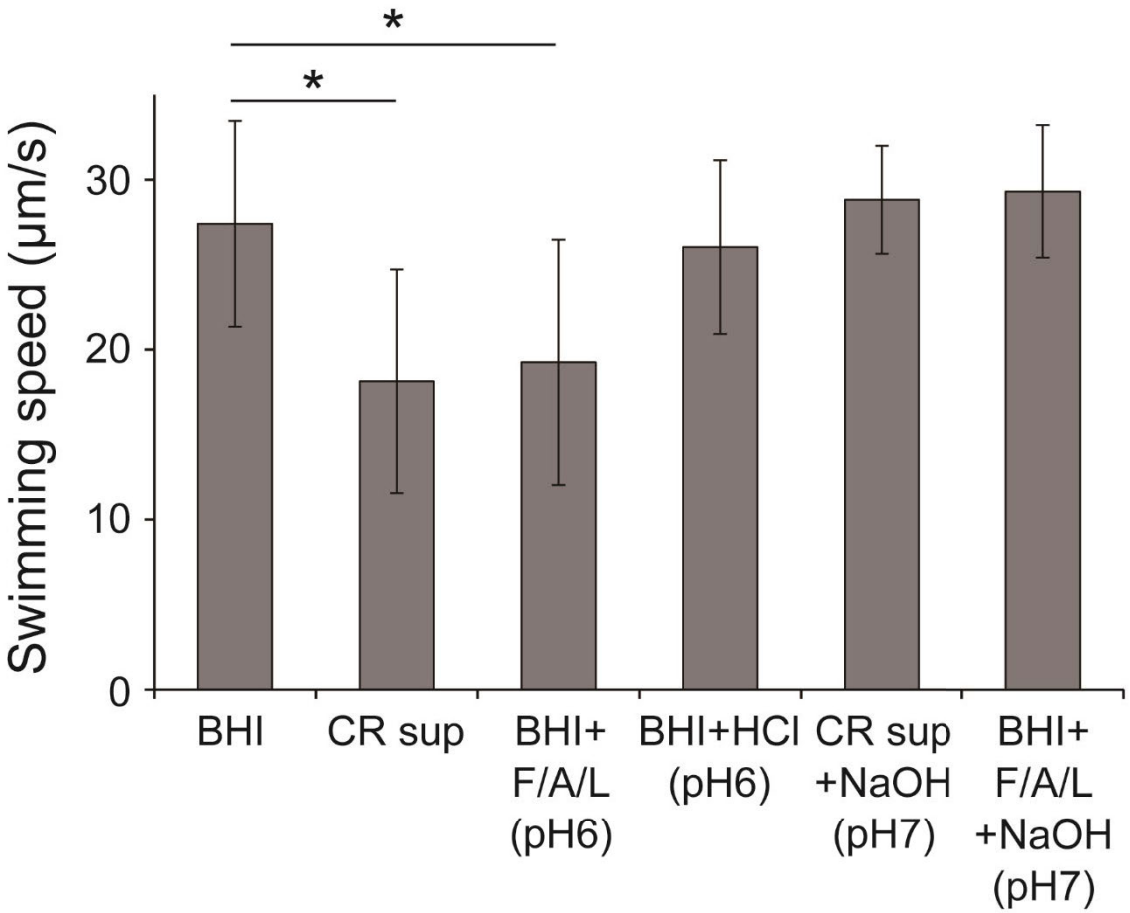


Fig. 2. Swimming speed of EHEC. Exposure to CR sup (pH 5.9~6.0) significantly decreased the swimming speeds of EHEC cells: 27.4 ± 6.1 μm s^{-1} (n = 38) in BHI and 18.1 ± 6.6 μm s^{-1} (n = 67) in CR sup. Swimming speeds measured in BHI adjusted to pH 6.0 by the addition of HCl (BHI+HCl) were comparable to those in fresh BHI: 26.0 ± 5.1 μm s^{-1} (n = 33 cells). Addition of formate, acetate, and lactate (BHI+F/A/L), three major acids produced by *C. ramosum* (Koyanagi et al., submitted), to BHI and pH adjustment to 6 decreased the swimming speeds of EHEC cells to the same level as that in CR sup: 19.3 ± 7.2 μm s^{-1} (n = 83 cells). When the pH values of CR sup and BHI+F/A/L were adjusted to 7 by the addition of NaOH, inhibition of motility was not observed. These results suggest that the inhibition of EHEC motility is attributed to the production of organic acids and acidification of media by *C. ramosum* fermentation. (Abbreviations: CR sup: *Clostridium ramosum* supernatant; F: formic acid; A: acetic acid; L: lactic acid).

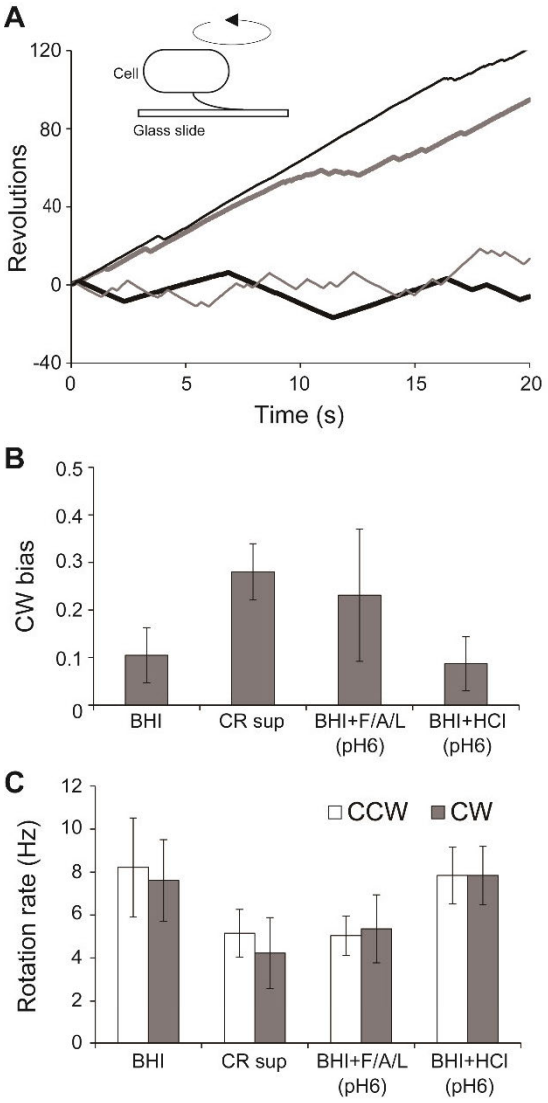


Fig.3. Effects of *C. ramosum* fermentation products on the rotation of EHEC flagella. Effect of CR sup on the rotation of the EHEC flagellum was examined by using the tethered cell assay (Fig. 3A, inset). In BHI, cells tethered on a glass surface via a single flagellum showed CCW-biased rotation (Fig. 3A, gray thick line). Exposure to CR sup remarkably increased motor reversal, biasing the direction of motor rotation to CW (Fig. 3A, black thick line). A time fraction of CW rotation (CW bias) was about 0.15 and 0.5 in BHI and CR sup, respectively (Fig. 3B). Both CCW and CW rotations were slowed by exposure to CR sup (Fig. 3C). BHI+HCl (pH 6.0) did not affect the tethered cell rotation, whereas BHI+F/A/L (pH 6.0) decreased rotation rate and increased reversal frequency. These results are consistent with those of the swimming assay, suggesting that flagellar rotation is disturbed by CR sup; the existence of organic acids under acidic conditions resulted in anomalies in swimming motility.

	BHI	CR sup	BHI+F/L/N	BHI+HCl
Flagellar length (μm)	5.3 ± 1.1	3.2 ± 1.1*	3.7 ± 0.9*	5.4 ± 1.2
Flagellar number/cell	6.1 ± 2.4	5.8 ± 1.9	5.9 ± 3.0	6.4 ± 2.0

Table 1. Flagellar number and length of EHEC grown in the absence and presence of *C. ramosum* supernatant or organic acids. The flagellar filament of EHEC consists of flagellin, and monomers of the protein are secreted by the type III secretion system (T3SS). The proton motive force (PMF), the sum of Δψ and ΔpH, is the driving force for not only flagellar rotation but also T3SS. A change in Δψ or ΔpH, even as the total value of PMF is maintained, affects protein secretion through T3SS, suggesting that T3SS discriminates between the two energy components. These facts raise the possibility that CR sup affects the synthesis of flagella in EHEC. EHEC cells were cultivated in the presence and absence of CR sup and compared the number and length of the flagella. Table 1 shows that the flagella were significantly shortened when the cells were grown in the presence of CR sup. As predicted from the results of the pH measurement, EHEC flagella were also shortened in BHI+F/A/L but not in BHI+HCl. The number of flagella per cell did not depend on culture conditions.

CHAPTER 3. Bacterium-Host

Involvement of adhesion and crawling behavior in *Leptospira* infection and host-preference

Leptospirosis is a zoonotic disease caused by pathogenic strains of the spirochete *Leptospira* (13). It has been reported worldwide, affecting accidental hosts, such as livestock, companion animals, and humans, with mild to fatal symptoms (14). The transmission of leptospirosis involves a *Leptospira* life cycle that has been adapted to mammalian hosts, commonly, such as the rat, in which the bacteria colonize the kidneys, are shed in the urine. Susceptible hosts are infected via contact with contaminated water by dermal abrasions, mucous membranes, and conjunctivae (15, 16). As silent-carrier animals, they maintain the bacteria and transmit leptospirosis to other animals. Maintenance hosts can be carriers for months to years or even a lifetime. Although the percutaneous infection of *Leptospira* results in colonization in the kidney or clinical symptoms in the host dependent manner, crucial factors for *Leptospira* serovars to determine the preferred host remain unclear. Meanwhile, the motility of *Leptospira* is an essential factor to enhance pathogenicity (8). *Leptospira* cells can smoothly swim when the anterior end is spiral shaped, and the posterior end is hook-shaped (Spiral–Hook) (Fig. 4). Swimming motility is well known as their prior way of migration, whereas recent studies (17, 18) showed that *Leptospira* possesses a “crawling” movement on solid surfaces. The model of *Leptospira* infection scheme proposed by Miyahara et al (19) stated that the migration of *Leptospira* from sinusoid to bile canaliculi through paracellular routes causes jaundice in *Leptospira* infected hamsters, In combined with our working model (Fig.5), we inferred involvements of adhesivity and crawling on host cells during the invasion are crucial to the enhancement of *Leptospira* infection. To verify the hypothesis, we examined the adhesivity and crawling motility of *Leptospira*

serovars on animal cells (Fig. 6, 7). The results (Fig. 8) raise the possibility that adhesivity and crawling motility are involved in the host preference of *Leptospira* serovars. Kinetics of bacterial cells could be one of the factors during the early stage of infection as well.

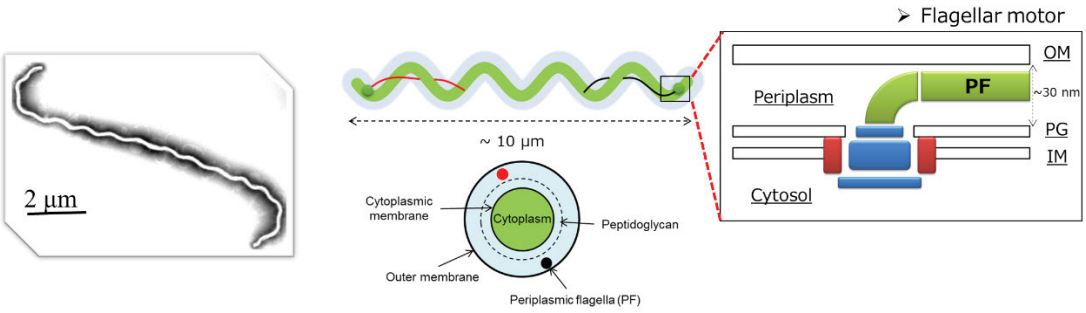


Fig.4. Transmission electron micrograph (magnification c.a.×3000) and a brief illustration of *Leptospira* cell structure. Two bacterial flagella are beneath the outer membrane.

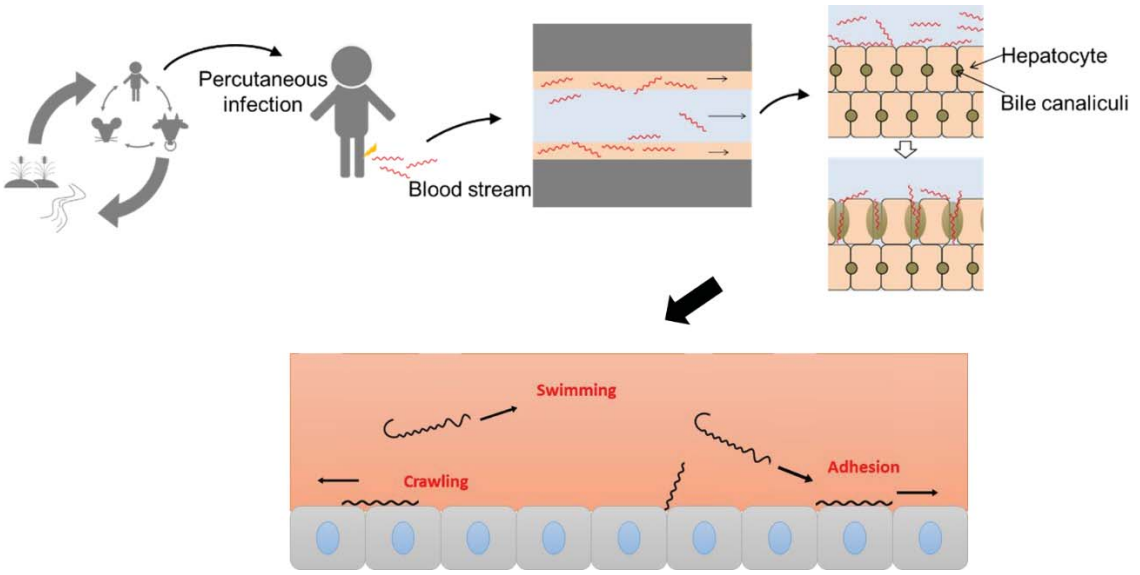


Fig.5. Brief schematic of the transmission of leptospirosis: motility is a virulence factor; *Leptospira* cells are actively moving in various form. (modified from the invasion model of Miyahara et al (19)).

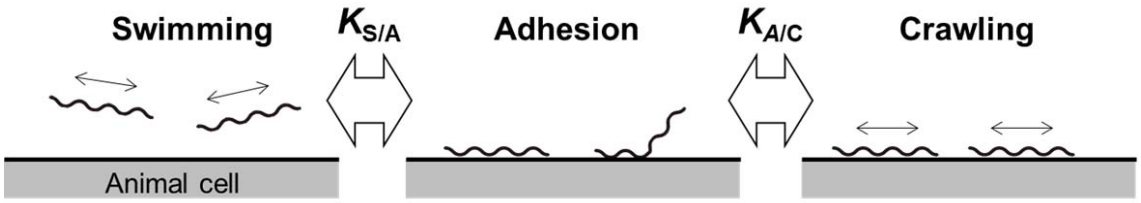


Fig.6. Moving pattern of *Leptospira* cells in the liquid medium and on the animal cell surface in the experimental observation. *K* indicates the equilibrium constant between each state.

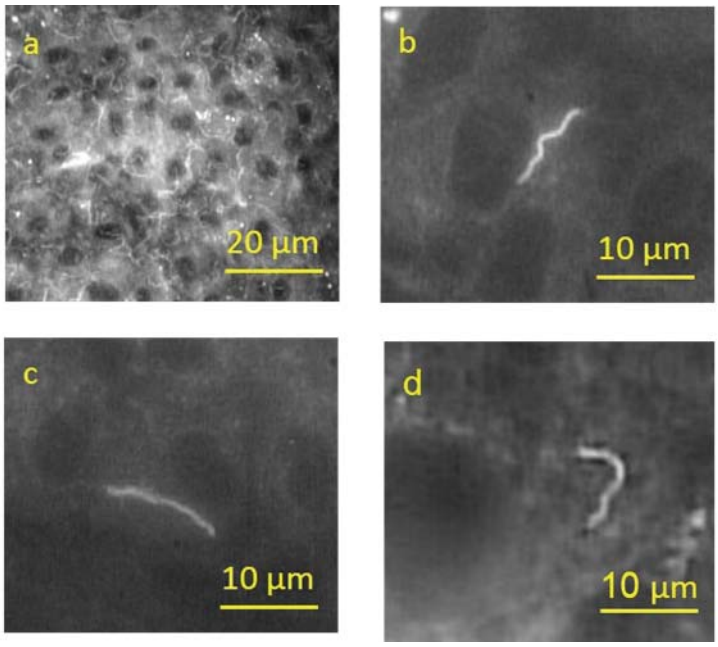


Fig.7. Actual images of different *Leptospira* serovar cells crawling on the animal cell (MDCK) sheet. *L. interrogans* serovar Icterohaemorrhagiae (a,b), *L. interrogans* serovar Manilae (c) and *L. biflexa* serovar Patoc (d).

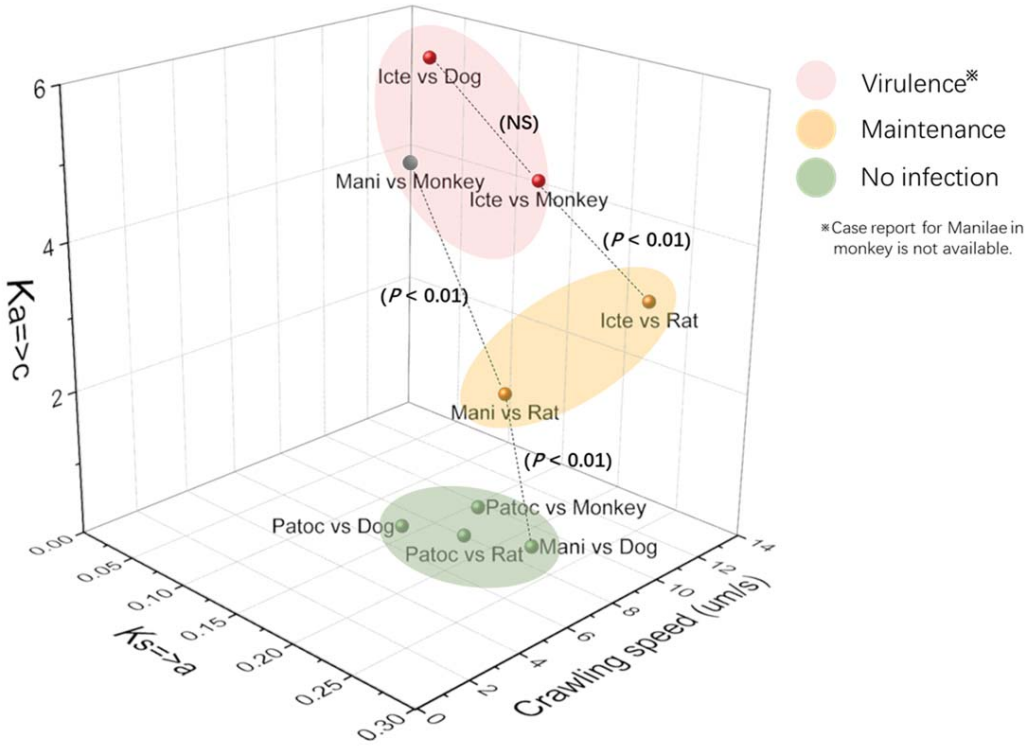


Fig.8. The three-dimensional scatter plot between the crawling speed and two equilibrium constants. serovar Icterohaemorrhagiae possesses remarkable overall score on adhesivity and crawling motility on both dog and monkey kidney cell. Manilae on rat and Icterohaemorrhagiae on rat cells shows middle scores in the plot. Combination of Manilae vs dog and the non-pathogenic serovar Patoc show relatively low scores. The colored area indicates the most common clinical outcomes of each combination.

CHAPTER 4. Bacterial response-Environmental stimuli

Photoresponsivity in the spirochete *Leptospira*

Environmental stimuli for bacteria are various in form. Chemotaxis is an example of bacterial movement in response to a chemical stimulus (22), while physical stimulus such as temperature, viscosity, and light (17) are also the significant to bacteria's life. As mentioned in chapter 1, this topic will focus on the response of bacterium to the essential environmental factor. Precisely on the photoresponsivity of a newly isolated *Leptospira* species now taxonomically known as *L. kobayashii*. The bacterium was first reported in 2018 (20). It shows a light-dependent modulation of swimming motility. (Fig. 9). As described in chapter 2, a *Leptospira* cell possesses a short-pitch helical cell body and two periplasmic flagella beneath the outer membrane, one at each cell end. *Leptospira* cells swim smoothly in liquid with an asymmetric morphology, i.e., when the anterior and posterior ends are spiral- and hook-shaped, respectively. The morphology of the cell ends frequently change during swimming. When both ends of the cell body exhibit hook- or spiral-shape, the cell moves neither forward nor backward. *L. kobayashii* recognizes red and blue-green lights and their intensities, resulting in distinct changes in motility: Enhancement of red light accelerates flagellar rotation, but the cell cannot translate because of remained symmetric morphology; blue-green light induces asymmetric cell morphology, allowing the cell to swim smoothly (Fig.11). Experiments suggest the contribution of rhodopsin-like sensors to the photoresponsivity. The genus *Leptospira* consists of four clades, of which the species in the same clade as *L. kobayashii* showed light-controlled motility in the study (Fig. 10). Although Bacteriorhodopsins are found with archaea widely (23, 24), the discovery of rhodopsin-like sensors in *Leptospira* provide a new insight on the molecular evolution in photobiology. It could contribute to the developments of new optogenetics in the future.

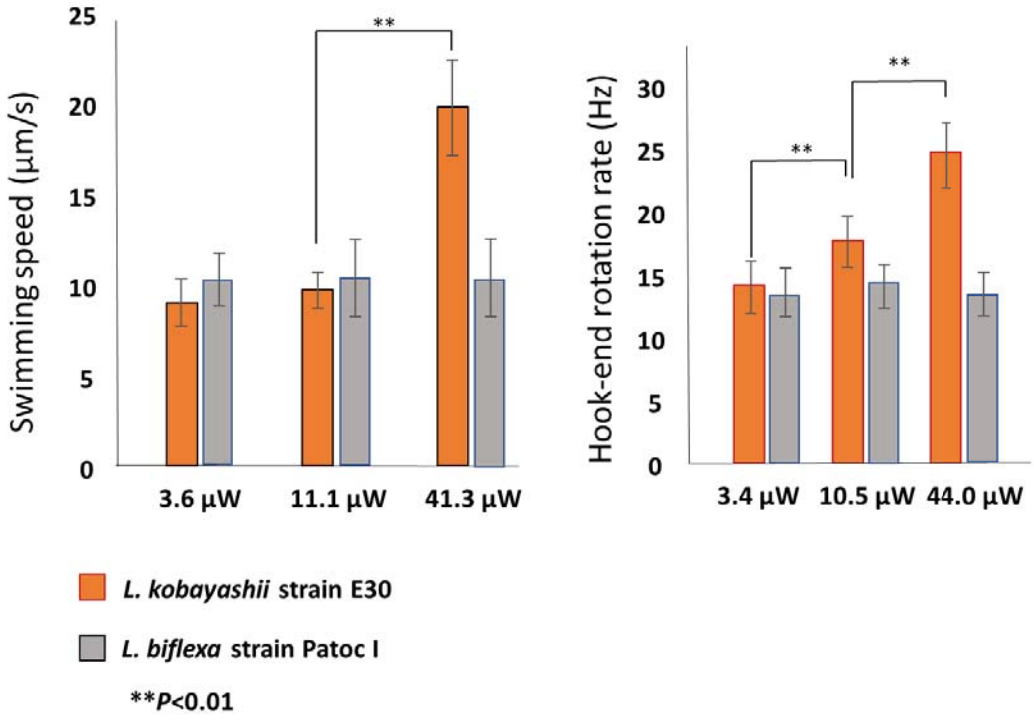


Fig.9. Motility of *Leptospira kobayashii* and *Leptospira biflexa* under different light-intensity. The newly found *L. kobayashii* shows increasing swimming speed and cell body rotation rate while the light power increased. The well-studied *L. biflexa* shows no response.

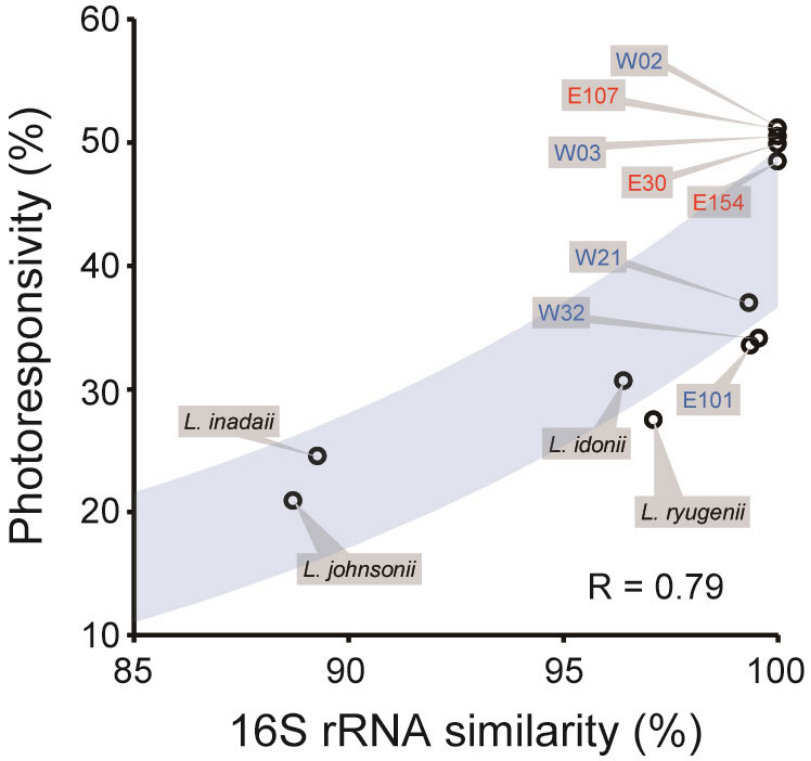


Fig.10. Pairwise plot between the degree of photoresponsivity and the 16s rRNA similarity of each *Leptospira* species or species which are confirmed with photoresponsivity. These species or strains are classified as the "photoresponsivity clade" in the *Leptospira* phylogenetic tree in this study.

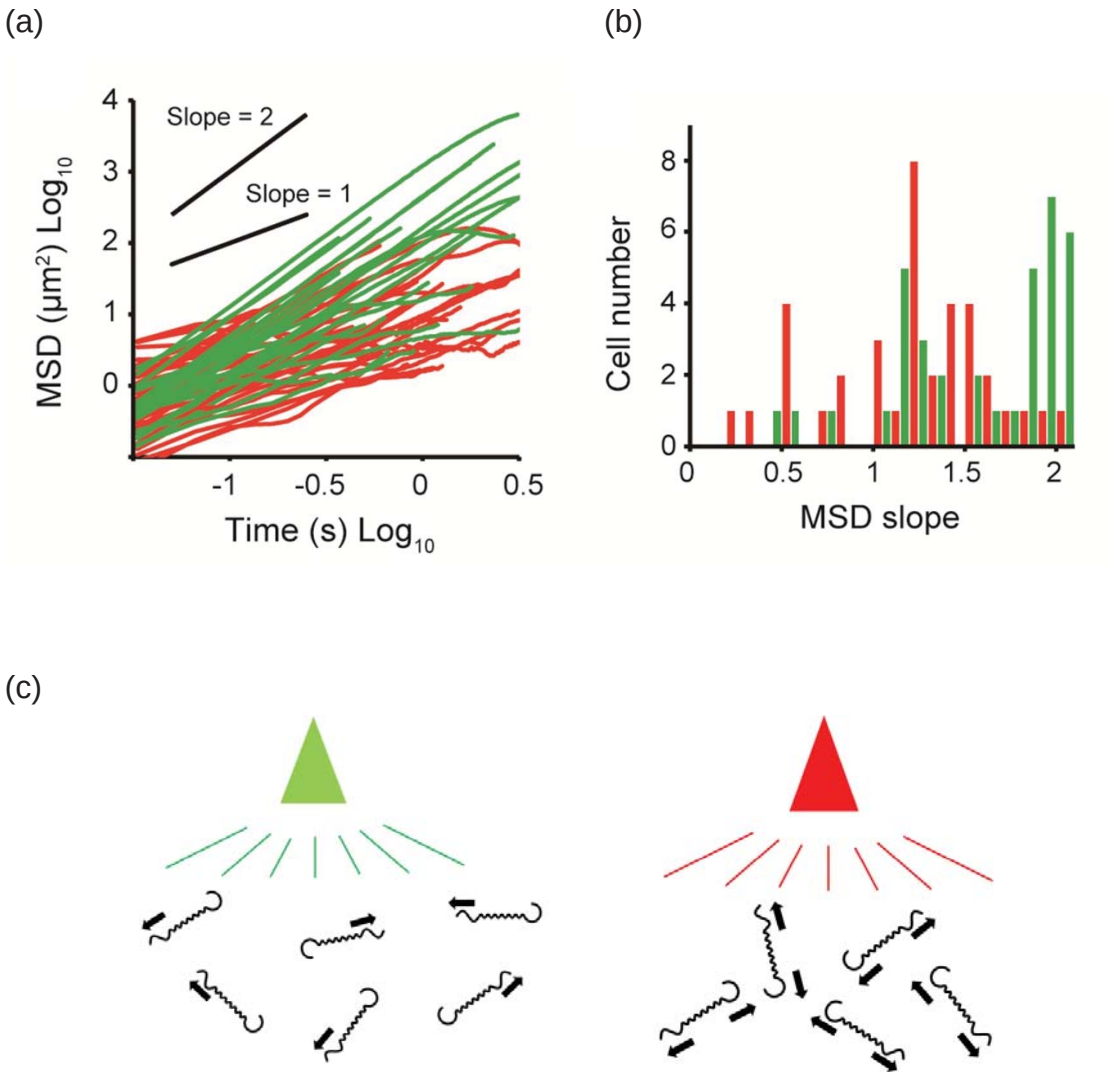


Fig.11. Different response of *L. kobayashii* to green and red light. (a) the mean square displacement (MSD) plot and (b) the fraction of the slopes indicates that *L. kobayashii* cells show better persistence of single-directional movement under the green light. (c) Brief schematic of cells movement under green and red light. Enhancement of red light accelerates flagellar rotation mostly results in the frequent switches on the moving direction, while blue-green light induces asymmetric cell morphology, allowing the cell to swim more smoothly.

CHAPTER 5. Conclusion

We discussed the bacterial motility via three topics. The main results and findings are described below:

1. The fermentation products of *C. ramosum* decreased the intracellular pH of EHEC, thereby interfering with flagellar rotation and swimming and the cells showed reduced motility. Synthesis of flagellar filament was also affected negatively whereas the formation of the fundamental structure of flagellar motor stood firm.
2. Crawling is the prior motility once *Leptospira* cells move on top of the animal cells. However, the crawling motility differed substantially through the *Leptospira* serotypes and the corresponding host species. Overall, the crawling motility with a pair of serovar-host combination is somehow consistent with the host-preference in leptospirosis to a certain degree.
3. The newly isolated species *Leptospira kobayashii* recognized red and green lights and their intensities, resulting in distinct changes in motility. Red light accelerated flagellar rotation, but the cell could not translate because of remained symmetric morphology. Green light induced asymmetric cell morphology, allowing the cell to swim smoothly. The species and strains in the same or near clade of *L. kobayashii* showed light-controlled motility.

Each of them reflects a scene while overall stands for the physiological processes in bacteria per se, including pathogenic versus commensal bacteria, bacteria against host and the response of bacteria to the environmental factor.

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論文審査の結果の要旨及び担当者

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学位論文題目	Studies on bacterial motility as a virulence factor and stimulation response （病原因子及び刺激応答としての細菌運動に関する研究）

論　文　審　査　の　結　果　の　要　旨

人類の歴史は、公衆衛生上の観点から微生物（細菌）との戦いであったと言っても過言ではない。細菌感染症は、ヒトを含む宿主動物の生体防御システムと病原細菌の相互作用の結果として起こる現象であり、感染が成立するためには病原細菌の病原因子が重要な役割を果たすことが知られている。病原因子として、宿主細胞への接着因子、宿主の免疫回避機構、毒素産生などさまざまな因子がこれまで研究され明らかとなってきている。多くの細菌は運動性をもっており、時々刻々変化する外部環境（栄養素、忌避物質、温度、pH、光など）に呼応した遊泳すなわち走性を示す。この細菌の運動性も病原因子の一つと考えられている。

本研究において博士候補者である許駿君は、病原因子として重要な細菌の運動性を、(1)病原細菌・常在細菌間相互作用、(2)病原細菌・宿主間相互作用、(3)細菌の環境因子に対する挙動、という異なった 3 つの視点から捉え、これらの課題を基礎微生物学的なアプローチで取り組み以下の結果を得た。

(1)ヒト腸内細菌叢の一つである *Clostridium ramosum* の腸管出血性大腸菌 O157 (EHEC)に対する生育抑制効果は、本菌が産生する短鎖脂肪酸（ギ酸、酢酸、乳酸）が EHEC 細胞内の pH 低下を来し、その結果 EHEC がもつ鞭毛の回転方向のスイッチ変換頻度の上昇と回転速度の低下、それに伴う遊泳速度の減少が要因であることを明らかとした。

(2)レプトスピラ症は人獣共通感染症起因菌である *Leptospira* 科に属するグラム陰性のらせん状細菌が原因である。この群の細菌は 300 種を超える血清型が存在し多くの動物種に感染することが知られているが、その宿主特異性の原因は不明である。*Leptospira* の鞭毛は細菌の外膜と内膜の間隙に存在し、その回転を制御することによって菌体全体を回転させ遊走する。この *Leptospira* を各種動物細胞と共培養し細菌の運動性を、培地中での遊走、宿主細胞表面への接着、細胞表面上での徘徊運動という 3 つの異なる状態を高速度カメラで測定する新しいアッセイ系を確立し、そのダナミクスを各状態の平衡定数として評価した。その結果、細胞表面への接着とその後の徘徊運動が宿主選択性に重要な役割を果たしていることが強く示唆された。

(3) *Leptospira* 外膜の内側に存在する 2 本の鞭毛は菌体の両極に固定されており、この鞭毛が環境シグナルに応答して回転し、一方の極がらせん状、他方の極がフック状の形態となったときにスムースに遊走する。2018 年に新規に登録記載された *Leptospira kobayashii* の外部環境シグナルに対する運動性を研究する過程で、本菌が光感受性をもち、青緑色の光に応答してスムースに遊走し、赤色の光に応答して鞭毛の回転速度が上昇するが遊走できないことが明らかとなった。この応答性の要因として、赤色光を照射したときに *Leptospira* の両極の形状は対称（ともにらせん状、あるいはフック状）となることがその原因であること、また、この光感受性には少なくとも 2 つのロドプシン様の光受容体が関与することを明らかとした。

以上のように、許君は *Leptospira* の運動性に係わるメカニズムを基礎的な視点から研究し、宿主特異性を決める要因としての運動能の重要性と、本菌が新規な光応答性を示すことを発見した。従って、審査員一同、本研究者に博士(農学)の学位を授与するに値するものと認定した。